User Manual

Knockout™ Single Vector Inducible RNAi System User Manual

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Table of Contents

I. Introduction 3
II. List of Components 6
III. Additional Materials Required 7
IV. Protocol Overview 8
V. shRNA Oligonucleotide Design 9
A. Selecting shRNA Target Sequences 9
B. Design of the shRNA Oligonucleotides 9
C. Oligonucleotide Quality 9
VI. Cloning shRNA Oligonucleotides into pSingle-tTS-shRNA 10
A. Protocol: Preparing the pSingle-tTS-shRNA Vector for Cloning 10
B. Protocol: Annealing the Oligonucleotides 10
C. Protocol: Ligating the Annealed ds Oligonucleotides into pSingle-tTS-shRNA 11
D. Transform Competent Cells, Identify Recombinant Clones, Prepare DNA for Transfection 11
VII. Optimization Experiments 12
A. Protocol: Antibiotic Titration at Fixed Cell Density. 12
B. Protocol: Determine Optimal Plating Density. 12
C. Test Doxycycline-Inducible Gene Knockdown in Host Cells By Transient Transfection with pSingle-tTS-Anti-Luc and a Luciferase Expression Vector [Recommended] 13
VIII. Development of pSingle-tTS-shRNA Stable Cell Lines 14
A. Protocol: Transfection and Selection of pSingle-tTS-shRNA Stable Cell Lines 15
B. Protocol: Screening pSingle-tTS-shRNA Clones for Induction 15
IX. Working with Stable Inducible RNAi Cell Lines 16
A. Determination of Effective Concentrations of Dox 16
B. Loss of Regulation 16
X. Troubleshooting Guide 17
XI. References 19
Appendix A: shRNA Target Sequence Requirements 21
Appendix B: Vector Information 22

List of Figures

Figure 1. Mechanism of RNA interference. 3
Figure 2. Small hairpin RNAs (shRNAs) generated using an oligonucleotide DNA sequence. 4
Figure 3. The Knockout Single Vector Inducible RNAi System 5
Figure 4. Doxycycline-induced knockdown of lamin A/C in HeLa cells. 8
Figure 5. shRNA oligonucleotide sequence design 9
Figure 6. Sensitive doxycycline-induced knockdown of luciferase activity. 13
Figure 7. Flow chart for developing a pSingle-tTS-shRNA cell line 14
Figure 8. pSingle-tTS-shRNA Vector Map. 22
Figure 9. pSingle-tTS-Anti-Luc Vector Map. 23

List of Tables

Table I: Troubleshooting guide for the Knockout Single Vector Inducible RNAi System 17
Table II: Examples of published target sequences 21

I. Introduction

Manipulating the cellular process of RNA interference (RNAi) is an effective method for supressing the expression of a specific gene to study its function. RNAi pathways are activated by various forms of double-stranded (ds) RNAs that contain sequences which are homologous to the mRNA transcript of a target gene (Figure 1; for reviews see Hutvagner & Zamore, 2002; Hammond *et al.*, 2001; and Sharp, 2001). Short hairpin RNA (shRNA) transcripts adopt a stable stem-loop structure in solution; can be easily expressed from a cloned oligonucleotide template; and are a convenient and reproducible means of activating RNAi in mammalian cell lines (Figure 2; Brummelkamp *et al.*, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002; and Yu *et al.*, 2002).

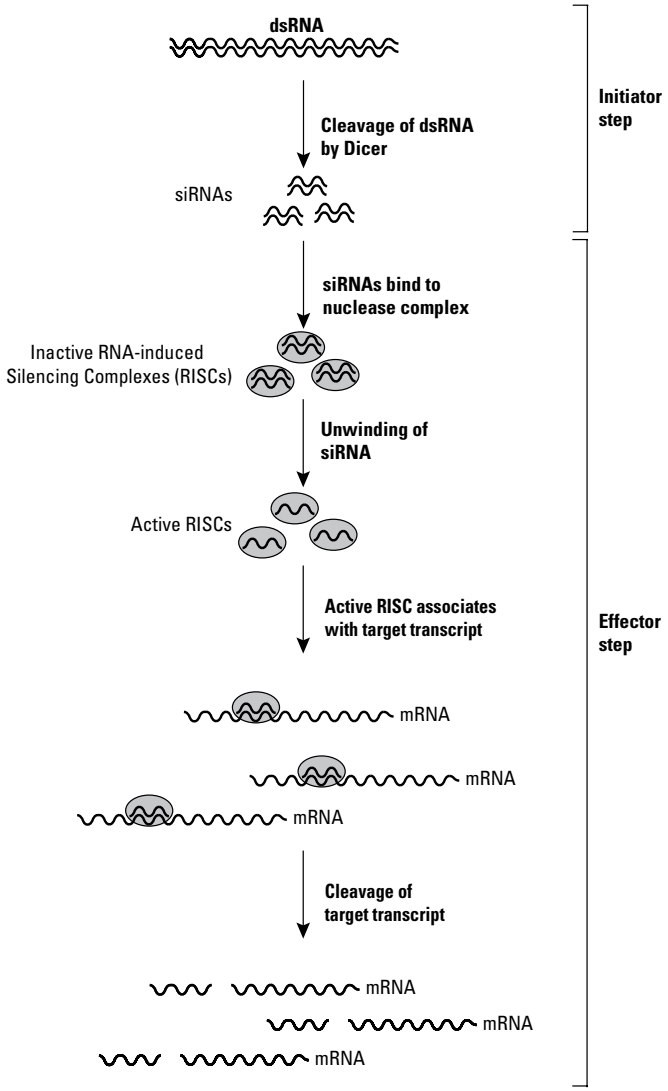

Figure 1. Mechanism of RNA interference. RNAi is activated by introducing a double-stranded RNA, whose sequence is homologous to the target gene transcript. The exogenous dsRNA is digested into 21–23 nucleotide (nt) small interfering RNAs (siRNAs), which bind a nuclease complex to form an RNA-induced silencing complex (RISC). The RISC then targets endogenous gene transcripts by base-pairing and cleaving the mRNA (for reviews, see Hammond et al., 2001; Sharp, 2001; and Huntvagner & Zamore, 2002). In contrast to genetic knockout methods, specific gene silencing is achieved quickly and easily in both animal and cell line models.

I. Introduction continued

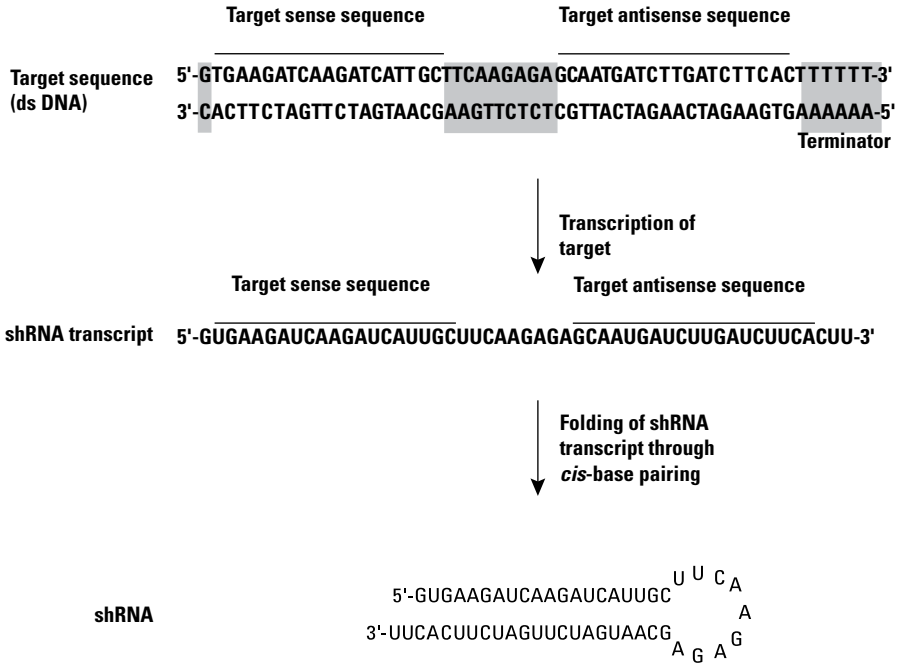

Figure 2. Small hairpin RNAs (shRNAs) generated using an oligonucleotide DNA sequence. This example shows a target sequence derived from the coding region of the ß-actin gene (Harborth et al., 2001). This target sequence is cloned downstream of a Pol III promoter in an expression vector for gene silencing in mammalian cells. A hairpin loop sequence is located between the sense and antisense sequences on each complementary strand. The shRNA behaves as an siRNA-like molecule capable of carrying out gene-specific silencing (Brummelkamp et al., 2002; Paddison et al., 2002; Paul et al., 2002; and Yu et al., 2002).

The **Knockout Single Vector Inducible RNAi System** allows you to quickly introduce and control the expression of functional shRNA molecules for the purpose of activating gene-specific RNAi (*Clontechniques*, October 2006). The tight on/off regulation of the system and coordinate inactivation of its target gene, are provided by a tetracycline-inducible system that responds to the presence of tetracyline or its more stable derivative, doxycycline (Dox) (Gossen & Bujard, 1992; Gossen, et al., 1993; Gossen, et al., 1995). The system features two essential components combined on its pSingle-tTS-shRNA vector: 1) CMV promoter/enhancer-controlled expression of the tetracycline-controlled regulatory protein, tTS and 2) a tetracycline-inducible shRNA expression cassette. The tTS protein is a powerful transcriptional suppressor created by fusing the Tet repressor protein (TetR) with a KRAB transcriptional silencing domain (Freundlieb, et al., 1999; Witzgall, et al., 1994; Wiznerowicz & Trono, 2003; *Clontechniques*, April 1999). The tetracyline-inducible, RNA Polymerase III (Pol III), hybrid promoter was created by linking a modified Tet-responsive element (TRE_{mod} from TRE-Tight; *Clontechniques,* April 2003) consisting of seven direct repeats of the *tet*O 19-mer from the *tet* operon, to a minimal human U6 snRNA promoter (Kunkel & Pederson, 1989).

I. Introduction continued

In the absence of the inducer, Dox, the tTS protein binds tightly to the *tet*O sequences within the TRE and actively silences transcription of the shRNA from the downstream minimal U6 promoter (Figure 3). In this basal state, background expression of the shRNA in the absence of induction is extremely low and prevents unwanted suppression of the target gene. When Dox is added to the culture medium, tTS dissociates from the TRE, relieving transcriptional suppression and permitting the shRNA to be transcribed from the U6 promoter. Once derepressed, the human U6 Pol III promoter provides high level expression in many cell types (Kunkel & Pederson, 1989), and the accumulating shRNA transcripts initiate RNAi-mediated suppression of the target gene. For more information on tetracycline-inducible gene expression systems, consult Gossen, et al., 1993 and Gossen, et al., 1994.

This user manual provides protocols for generating an inducible RNAi system in any cell line. It covers the design and cloning of shRNA oligonucleotides into the pSingle-tTS-shRNA vector; pilot studies to characterize the system for your host cell line; and delivering the system into your cells. A complete, inducible RNAi system can thus be established in any cell line after a single round of transfection and subsequent selection of stable clonal cell lines.

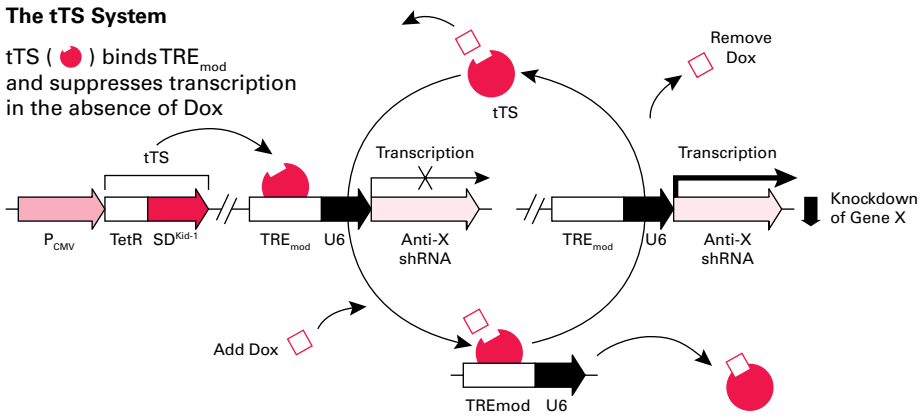

Figure 3. The Knockout Single Vector Inducible RNAi System uses a modified form of the tightly regulated, tetracycline-controlled gene expression system. In the absence of Dox, tTS binds the *tet*O sequences within the TRE/U6 promoter and actively silences transcription of the shRNA. When Dox is added to the culture medium, tTS dissociates from the TRE, relieving transcriptional suppression and allowing high level transcription of the shRNA from the hybrid TRE/U6 promoter.

II. List of Components

Store all kit components at –20°C:

Knockout Single Vector Inducible RNAi System (Cat. No. 630933)

- 20 µg pSingle-tTS-shRNA Vector (500 ng/µl)
- 20 µg pSingle-tTS-Anti-Luc Control Vector (500 ng/µl)
- 50 ml Tet System Approved FBS

Supporting Documents

- Knockout Single Vector Inducible RNAi System User Manual (PT3922-1)
- pSingle-tTS-shRNA Vector Information Packet (PT3923-5)
- pSingle-tTS-Anti-Luc Vector Information Packet (PT3924-5)

Visit our website at **www.clontech.com** for a current list of products available for RNAi.

III. Additional Materials Required

The following materials or their equivalents are required but not supplied:

For cloning shRNA oligonucleotides

- T4 DNA Ligase and 10X buffer (i.e. New England Biolabs, Cat. No. M0202S)
- Nuclease-free deionized H_2O
- Fusion-Blue™ Competent Cells (Cat. No. 636700)

Plasmid DNA purification

To ensure sufficient DNA purity for efficient transfections, prepare all plasmids by using NucleoBond or NucleoBond Xtra technology, or by CsCl density gradient purification (Sambrook et al., 2001).

- NucleoBond® Plasmid Maxi EF Kit (Cat. No. 635953)
- NucleoBond® Xtra Midi and Maxi Kits (Cat. Nos. 637101 & 637106)
- NucleoSpin® Multi-8 Plus Plasmid Kit (Cat. No. 635976)
- NucleoSpin® Extract II Kit (Cat. No. 636971)

Supplements for cell culture and transfections

In addition to the basic supplies and skills required to culture the cell line of interest, users will need the following to complete the protocols in this user manual.

- G418 (Cat. No. 631307). For selection of stable cell lines that contain integrated copies of the pSingle-tTS-shRNA vector. This antibiotic is supplied as a powder containing approximately 0.7 g G418 per gram of powder. Make a 10 mg/ml stock solution by dissolving 1 g of G418 powder in approximately 70 ml of medium (without supplements). Filter sterilize and store in small aliquots at –20°C.
- Cloning cylinders (PGC Scientific Cat. Nos. 62-6150-40, -45, -12, & -16)
- Doxycycline (Cat. No. 631311), for shRNA induction. Dilute to 1–2 mg/ml in H_2O, filter sterilize, aliquot, and store at –20°C in the dark. Use within one year.
- CLONfectin™ Transfection Reagent (Cat. No. 631301)
- CalPhos™ Mammalian Transfection Kit (Cat. No. 631312)

Luciferase assay

- Luciferase expression vector. We recommend the pGL2-Control Vector (Promega, Cat. No. E1611) for SV40 promoter/enhancer controlled expression of luciferase.
- Luciferase Assay System (Promega, Cat. No. E1500); luminometer is also needed.

Gene-specific assays

When testing your pSingle-tTS-shRNA construct for functionality, you will need a gene-specific assay to test for the suppression of your target gene. Examples of such assays include:

- Western blot using an antibody to the protein product.
- RT-PCR using specific primers. Ensure that you can discriminate between PCR products generated from mRNA and those derived from genomic DNA.
- Northern blot using a gene-specific probe.
- Functional assay for the protein product.

IV. Protocol Overview

PLEASE READ ALL PROTOCOLS IN THEIR ENTIRETY BEFORE BEGINNING.

Successfully implementing the Knockout Single Vector Inducible RNAi System consists of performing the steps listed below, all of which are included in this user manual.

1. Select appropriate mRNA target sequences for the gene of interest.
2. Design and synthesize the shRNA oligonucleotides corresponding to the mRNA target(s).
3. Anneal the shRNA oligos, and clone them into the XhoI/HindIII-digested pSingle-tTS-shRNA vector.
4. Identify recombinant vector clones; propagate and purify the vector DNA for transfection.
5. Transfect the recombinant pSingle-tTS-shRNA vector into target cells and select for stable transformants with G418.
6. Pick colonies of G418-resistant clones (≥30 each), expand the cell lines for screening.
7. Identify clones that demonstrate inducible knockdown. Freeze the best responders for long-term storage.

Expected Results

When the Knockout Single Vector System was used for inducible knockout of endogenous lamin A/C gene expression in HeLa cells, exposing the cells to Dox reduced lamin A/C protein levels to near undetectable levels after 72 hr (Figure 4, Panel A). Treating the cells with increasing concentrations of Dox demonstrated a dose-dependent reduction of lamin A/C (Figure 4, Panel B).

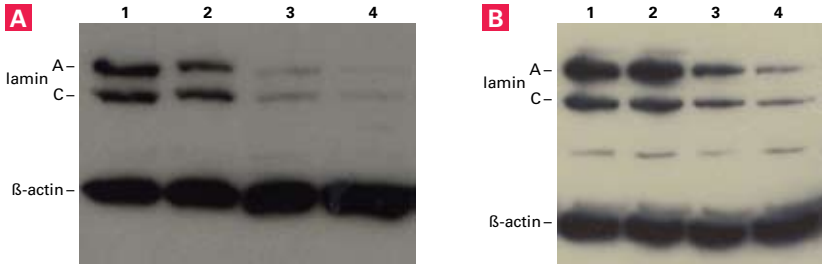

Figure 4. Doxycycline-induced knockdown of lamin A/C in HeLa cells. Panel A. A stable HeLa cell line that expresses an anti-lamin A/C shRNA was produced using the Knockout Single Vector System. Cells were grown in the absence or presence of Dox (1 µg/ml)for the times indicated. They were then harvested and analyzed by Western blot using ß-actin expression as a control. Suppression of lamin A/C expression is evident after 6 hr of treatment, and is virtually abolished after 72 hr. Lane 1: control. Lanes 2–4: 6 hr, 48 hr, and 72 hr, respectively. **Panel B.** The anti-lamin A/C HeLa cell line was grown in the absence or presence of increasing concentrations of Dox for 72 hr, then harvested and analyzed by Western blot. Lane 1: parental cells. Lane 2–4: 0, 0.1, and 1 µg/ml Dox, respectively.

V. shRNA Oligonucleotide Design

A. Selecting shRNA Target Sequences

The degree to which a target gene is knocked down depends largely on choosing ideal target sequence(s) within your gene of interest, and on properly designing the corresponding shRNA oligonucleotides. For users unfamiliar with the requirements of successful mRNA target sequences, we have provided some guidelines for identifying them in Appendix A and on the *Bioinformatics-siRNAdesigner* page in the "Online Tools" section of our website (**www.clontech.com)**. Further information can be found in Brummelkamp *et al.*, 2002; Paddison *et al.*, 2002; Paul *et al.*, 2002; and Yu *et al.*, 2002. In addition, we highly recommend that you test more than one shRNA sequence per gene of interest.

B. Design of the shRNA Oligonucleotides

It is necessary to synthesize two complementary oligonucleotides (a upper and lower strand) for each shRNA target site. Figure 5 illustrates the overall structure of the prototypical oligonucleotide sequences for use in the pSingle-tTS-shRNA vector. The sequences of the oligonucleotides should include the following:

1. A 5'-XhoI restriction site overhang on the top strand and a 5'-HindIII restriction site overhang on the bottom strand. These restriction sites will enable directional cloning of the annealed oligonucleotides into the XhoI/HindIII-digested pSingle-tTS-shRNA vector.
2. A guanine (G) residue added upstream of the 5'- end of the shRNA sense strand, if the target sequence does not start with a purine (preferred as Pol III transcription start site).
3. The 19-base target sense sequence; see Appendix A for sequence requirements.
4. A 7–9 nucleotide hairpin loop sequence. (We typically use 5'-TTCAAGAGA- 3'; see Sui *et al.*, 2002; Lee *et al.*, 2002; Paddison *et al.*, 2002; Brummelkamp *et al.*, 2002; and Paul *et al.*, 2002 for other effective loop sequences.)
5. The 19-base target antisense sequence.
6. A RNA Pol III terminator sequence consisting of a 5–6 nucleotide poly(T) tract.
7. Recommended, but not essential: a unique restriction site positioned immediately downstream of the terminator sequence for convenient restriction digest analysis to confirm the presence of the cloned insert. We suggest using Mlu I (5'-ACGCGT- 3') since this site does not exist in pSingle-tTS-shRNA.

Thus, beginning at the 5' end, a typical oligonucleotide for the upper strand should have 5 bases to complete the XhoI cloning site, additional G residue (if needed), 19 bases of sense sequence, 7–9 bases of hairpin loop, 19 bases of antisense sequence, 6 bases of terminator T residues, 6 bases of a unique restriction site (MluI), and a final A residue to complete the downstream HindIII cloning site at the 3' end.

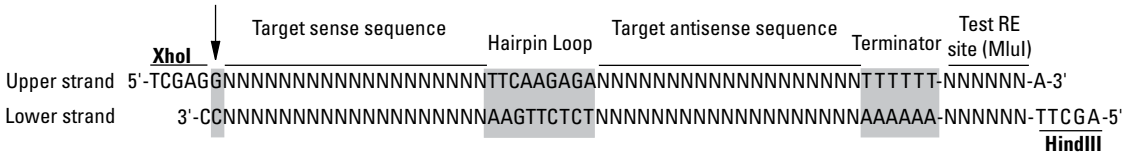

Figure 5. shRNA oligonucleotide sequence design. The arrow denotes the purine residue required for RNA Pol III to initiate transcription. The hairpin loop sequence shown is one of many functional loop sequences used to generate shRNAs. Termination is signaled using a poly(T) tract. Including a unique restriction site (Test RE site; i.e. MluI) allows confirmation of the cloned insert after the ligation and transformation reactions. XhoI (upper) and HindIII (lower) 5′ overhangs are necessary for directional cloning into the pSingle-tTS-shRNA vector. Visit the Bioinformatics-siRNAdesigner page in the "Online Tools" section of our website (www.clontech.com) and see Table I in Appendix A for examples of target sense and antisense sequences for selected genes.

C. Oligonucleotide Quality

Depending on the quality and degree of full-length, it is usually possible to clone the oligonucleotides without first gel-purifying them. In our hands, oligonucleotides less than 65-nt in length do not require PAGE purification, but this will depend on the synthesis and the manufacturer. If the oligonucleotides are to be gel purified, order them at the 200 nmol scale and gel purify them by standard methods. The use of phosphorylated oligonucleotides is not required.

VI. Cloning shRNA Oligonucleotides into pSingle-tTS-shRNA

Protocol
2-3 hr.

A. Protocol: Preparing the pSingle-tTS-shRNA Vector for Cloning

The annealed shRNA oligos will be inserted between the XhoI and HindIII sites in pSingle-tTS-shRNA. The vector actually contains two alternating sets of XhoI and HindIII sites at this insertion site (see Appendix B: Vector Information). Digestion with these enzymes cuts the intervening sequence into several smaller fragments that are easily removed by spin column purification.

1. Digest 1 µg of pSingle-tTS-shRNA with Xho I and Hind III restriction enzymes according to the manufacturer's protocol. These two enzymes typically cut in compatible buffers.
2. Purify the digested vector DNA using a spin column from the NucleoSpin Extract II Kit (Cat. No. 636971), or on an agarose gel using standard methods.
3. Store the purified vector DNA at –20°C until ready to ligate the annealed oligos.

Protocol
~15 min

B. Protocol: Annealing the Oligonucleotides

For convenience, Steps 3–6 can be performed in a thermal cycler.

1. Resuspend each purified oligonucleotide in TE buffer to a final concentration of 100 µM.
2. Mix the oligos for the top strand and the bottom strand at a ratio of 1:1. This mixture will ultimately yield 50 µM of ds oligo (assuming 100% theoretical annealing).
3. Heat the mixture to 95°C for 30 sec to remove all intramolecular secondary structure and disrupt the internal hairpin of each oligonucleotide. This promotes intermolecular annealing.
4. Heat at 72°C for 2 min.
5. Heat at 37°C for 2 min.
6. Heat at 25°C for 2 min.
7. Store on ice.

The annealed oligonucleotides are now ready for ligation into the pSingle-tTS-shRNA vector. Alternatively, the annealed oligonucleotides can be stored at –20°C until ready to use.

VI. Cloning shRNA Oligonucleotides into pSingle-tTS-shRNA continued

Protocol
1-3 hr.

Recipe

C. Protocol: Ligating the Annealed ds Oligonucleotides into pSingle-tTS-shRNA

1. Dilute the annealed ds oligo (from Step B.7) 100-fold with TE buffer to obtain a concentration of 0.5 µM.

   NOTE: To ensure good ligation efficiency it is necessary to dilute the oligo so that it only moderately exceeds the concentration of the vector DNA. Using a large excess of oligo will inhibit ligation.

2. Assemble a ligation reaction for each annealed pair of oligonucleotides by combining the following reagents in an microfuge tube:

1	µl	pSingle-tTS-shRNA vector DNA, XhoI/HindIII-digested (50 ng/µl)
1	µl	Diluted, annealed oligonucleotides (0.5 µM)
1.5	µl	10X T4 DNA ligase buffer
10.5	µl	Nuclease-free H_2O
1	µl	T4 DNA ligase (400 U/µl)
15	µl	Total volume

3. If desired, a control ligation can be assembled using 1 µl of nuclease-free H_2O instead of the annealed oligos.
4. Incubate the reaction mixture according to the ligase manufacturer's recommendations.

Protocol
~3 days.

D. Transform Competent Cells, Identify Recombinant Clones & Prepare DNA for Transfection

Fusion-Blue Competent Cells (Cat. No. 636700) are an *E.coli* K-12 strain that provides high transformation efficiency. The strain carries recA and endA mutations that make it a good host for obtaining high yields of plasmid DNA. We routinely use this strain for our shRNA cloning.

1. Transform competent *E. coli* with 2 µl of the ligation reaction, using the protocol supplied with the cells.
2. Plate different volumes (20–150 µl) from each transformation on LB agar + ampicillin plates (50-100 µg/ml). Incubate overnight at 37°C
3. Pick 4-8 well isolated colonies from each ligation/transformation and inoculate each into a small-scale liquid culture. Grow overnight at 37°C with shaking.
4. Prepare plasmid DNA minipreps. We recommend using our NucleoSpin Plasmid Kit (Cat. No. 636042).
5. Identify the desired recombinant plasmid by restriction analysis using the unique restriction site (i.e. MluI) within the shRNA oligonucleotide sequence. If desired, verify your insert by sequencing.

   NOTE: Since there is always a chance for mutations in the oligo due to synthesis errors, we strongly recommend that you sequence at least two clones to verify the correct oligo sequence. Because hairpin sequences are difficult to sequence, inform your sequencing facility so that sequencing conditions can be adjusted accordingly.

6. Once a positive clone has been identified, make a large-scale DNA prep of the recombinant pSingle-tTS-shRNA vector. To ensure optimal purity of the DNA for transfection, using a NucleoBond or NucleoBond Xtra Kit, or CsCl density gradient purification (Sambrook *et al.*, 2001).

VII. Optimization Experiments

Titrating Antibiotics and Plating Density for Selection (Kill Curves)

Prior to using the G418 antibiotic to establish stable cell lines, you must first determine the optimal concentration for selection in the target host cell line. Lot-to-lot variations in potency exist for G418, so each new lot should be titrated. Perform two experiments: (1) a titration to determine optimal drug concentration, and (2) a determination of the optimal plating density.

Protocol
1–2 weeks

A. Protocol: Antibiotic Titration at Fixed Cell Density.

For selecting stable transformants, use the lowest concentration that begins to result in massive cell death in ~5 days and kills all the cells within two weeks. For HeLa, HEK 293, MCF7, and CHO cells, we have found 400–500 µg/ml G418 to be optimal.

1. Plate 2 x 10^5 cells in each of six 10 cm tissue culture dishes containing 10 ml of the appropriate complete medium plus varying amounts (0, 50, 100, 200, 400, and 800 µg/ml) of G418.
2. Incubate the cells for 5–14 days, replacing the selective medium every four days (or more often if necessary).
3. Examine the dishes for viable cells every two days.

Protocol
1–2 weeks

B. Protocol: Determine Optimal Plating Density.

When selecting stable transfectants, use a plating density that allows the cells to reach ~80% confluence before massive cell death begins (at about day 5). This is the cell density at which cells should be plated for selection of stable transfectants. For HeLa cells, we have found 2 x 10^5 cells/10 cm dish to be a good plating density.

Once you have determined the optimal drug concentration, determine the optimal plating density by plating cells at several different densities in the presence of a constant amount of drug. If cells are plated at too high a density, they will reach confluence before the selection takes effect. Optimal plating density is dependent on population doubling time and cell surface area. For example, large cells that double rapidly have a lower optimal plating density than small cells that double slowly.

1. Plate cells at several different densities in each of six 10 cm tissue culture dishes containing 10 ml of the appropriate selective medium. Suggested densities (cells/10 cm dish): 5 x 10^6, 1 x 10^6, 5 x 10^5, 2 x 10^5, 1 x 10^5, and 5 x 10^4.
2. Incubate the cells for 5–14 days, replacing the selective medium every four days.
3. Examine the dishes for viable cells every two days.

Important information about Tet System transfections

Transient and stable transfections can be performed using various methods with efficiencies that vary greatly for different cell lines. Therefore, when working with a cell line for the first time, it is useful to compare the efficiencies of several transfection methods in transient assays.

- CalPhos and CLONfectin produce reliable results for either calcium-phosphate or liposome-mediated transfections, respectively. Other similar reagents, or electroporation methods, may also work well for the cell line in question.
- Optimize transfection efficiency with a noninducible reporter/expression vector, such as pACGFP1-N1 (Cat. No. 632469) for fluorescent protein expression or the pGL2-Control Vector from Promega (Cat. No. E1611) for SV40 promoter/enhancer controlled expression of luciferase. Use the Promega's Luciferase Assay System (Cat. No. E1500) to measure luciferase activity.
- Once a preferred method of transfection is identified, it may be necessary to optimize parameters such as cell density, the amount and purity of the DNA, media conditions, and transfection time. Once optimized, these parameters should be kept constant to obtain reproducible results.

VII. Optimization Experiments continued

Protocol
3 days.

C. Test Doxycycline-Inducible Gene Knockdown in Host Cells By Transient Transfection with pSingle-tTS-Anti-Luc and a Luciferase Expression Vector [Recommended]

Tet expression systems have been established in numerous cell lines including HeLa, MCF7, and HEK 293. Performing inducible knockdown in a transient assay by using the pSingle-tTS-Anti-Luc control vector and a luciferase expression vector (we recommend pGL2-Control from Promega) will provide a quick indication of the Knockout Inducible RNAi System behaves in your particular cell line.

Using your optimized transfection method, transfect cells using different ratios of the luciferase expression vector to the pSingle-tTS-Anti-Luc control vector, with and without induction by Dox. As a starting point you can try them at 1:1 or 1:2, respectively. For example:

Transfection	Luciferase Vector (i.e. pGL2-Control)	pSingle-tTS-Anti-Luc	Null DNA (i.e. pSingle-tTS-shRNA)
Luciferase Control	1	0	2
Luc + Anti-Luc (1:1)	1	1	1
Luc + Anti-Luc (1:2)	1	2	0

1. Using two 6-well plates, seed cells in enough wells to transfect the following vector ratios in duplicate, with and without Dox:
2. Depending on the transfection method, adjust the Dox concentration in half of the wells to 1 µg/ml Dox by adding a volume of 100 µg/ml stock solution of Dox (100X) equivalent to 1/100 the total culture volume, or replace the transfection medium with fresh medium containing Dox.

   NOTE: Be sure to culture the cells using Tet System Approved FBS or serum shown to be free of tetracycline activity.
3. Harvest cells after 48–72 hr, and assay for luciferase activity.

   NOTE: Knockdown levels are almost always lower in transient assays than in properly screened clonal stable cell lines. Therefore, an apparent lack of induction response in the transient assay should not be the sole reason for aborting your experiments in a particular cell line.

Expected Results

When using HEK 293 transfected with a 1:1 ratio of pSingle-tTS-Anti-Luc control vector and a luciferase expression vector (pCMV-Luc), we consistently observe a 60-75% knockdown of luciferase activity within 72 hr of 1 µg/ml Dox induction as shown in Figure 6.

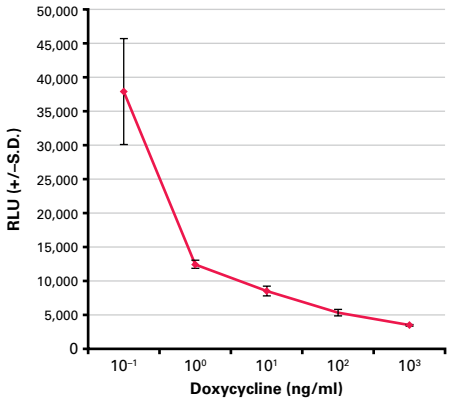

Figure 6. Sensitive doxycycline-induced knockdown of luciferase activity. HEK 293 cells were transiently cotransfected with the pSingle-tTS-shRNA vector expressing an anti-luciferase shRNA and a pCMV-luciferase expression vector at a ratio of 1:1. Cells were grown in the absence or presence of 0–1 µg/ml Dox for 72 hr, then harvested and lysed to measure luciferase activity. Luciferase activity was reduced by 67% at 1 ng/ml Dox and by 88% at 1 µg/ml.

VIII. Development of pSingle-tTS-shRNA Stable Cell Lines

Important Considerations

The following protocol describes the development of cell lines containing copies of your recombinant pSingle-tTS-shRNA vector stably integrated into the cellular genome. The goal is to generate a clonal cell line that exhibits normal levels of your gene of interest in the absence of Dox, but experiences significant reduction (knockdown) of this gene product in the presence of Dox at ≤1 µg/ml.

The transfection and selection protocols must be optimized for each cell type (see Section VII). Parameters subject to optimization include: plating density, transfection method, the G418 concentration used for selection, and growing times prior to clone selection.

The site where the pSingle-tTS-shRNA vector integrates may profoundly affect the expression level of the tTS silencer and hence, induction of the shRNA. For these reasons, we recommend that as many clones as possible be isolated at Step 8 in the following Protocol A. Isolate enough colonies in order to *test* at least 30 clones, bear in mind that some clones may not survive isolation and expansion. Though it is possible that an optimal clone may be found by screening fewer colonies, an unsuccessful screen will cause significant delays.

ATTENTION: Do not work with a mixed population of clones as expression and induction will be neither reproducible nor dependable.

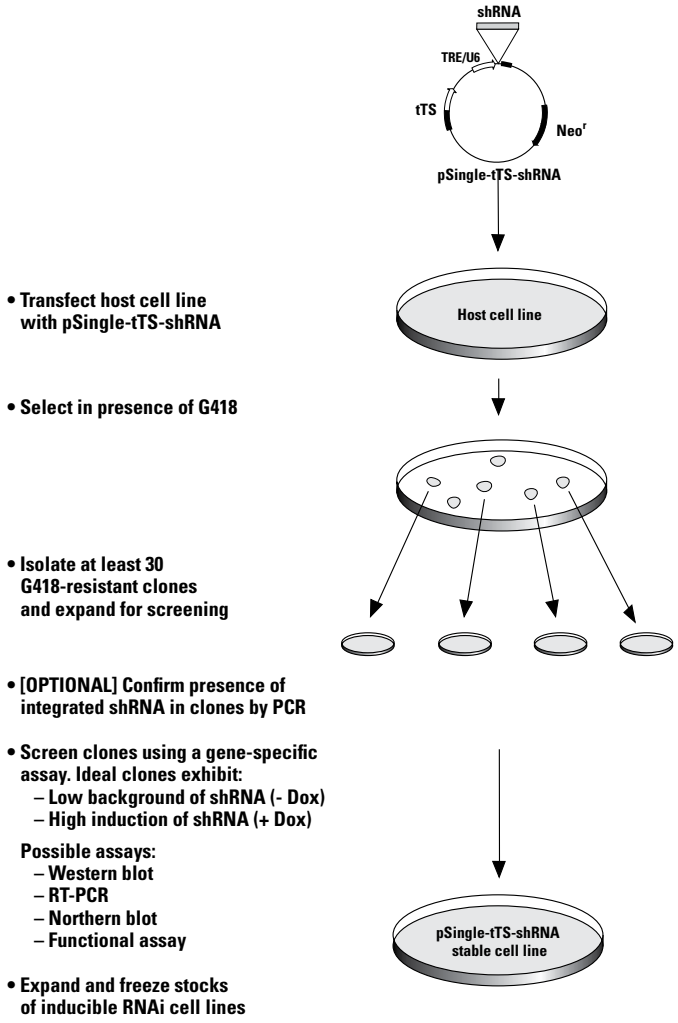
shRNA
TRE/U6
tTS
Neor
pSingle-tTS-shRNA
• Transfect host cell line with pSingle-tTS-shRNA
Host cell line
• Select in presence of G418
• Isolate at least 30 G418-resistant clones and expand for screening
• [OPTIONAL] Confirm presence of integrated shRNA in clones by PCR
• Screen clones using a gene-specific assay. Ideal clones exhibit:
– Low background of shRNA (- Dox)
– High induction of shRNA (+ Dox)
Possible assays:
– Western blot
– RT-PCR
– Northern blot
– Functional assay
pSingle-tTS-shRNA stable cell line
• Expand and freeze stocks of inducible RNAi cell lines

Figure 7. Flow chart for developing a pSingle-tTS-shRNA cell line

VIII. Development of pSingle-tTS-shRNA Stable Cell Lines continued

Protocol
2–4 weeks

A. Protocol: Transfection and Selection of pSingle-tTS-shRNA Stable Cell Lines

1. Grow cells to ~80% confluency in complete medium or to a density appropriate for your transfection method.
2. Transfect your recombinant pSingle-tTS-shRNA vector by the desired method.
3. Following the transfection incubation period, split the cells and plate them in ten, 10 cm culture dishes, each containing 10 ml of the appropriate complete medium (without G418), at the optimal density determined in Section VII.
4. Allow cells to divide twice (24–48 hr), then add G418 to the concentration determined in Section VII for selection. This is usually 400–500 µg/ml G418.
5. Replace medium with fresh complete medium plus G418 every four days, or more often if dead cells accumulate or if medium becomes depleted.
6. After about five days, cells that have not taken up the plasmid should start to die. If necessary, you can split the cells if they reach confluency before massive cell death begins. However, this should be avoided, since replating cells at this point may result in plates containing too many colonies for effective colony isolation.
7. After 2–4 weeks, isolated G418-resistant colonies should begin to appear.
8. Isolate large, healthy colonies and transfer them to individual plates or wells. Use care to prevent contamination of the cultures. Suspension cultures must be cloned using the limiting dilution technique. When working with adherent cells at Clontech, we generally isolate clones using cloning cylinders or cloning discs. Isolate as many clones as possible, typically enough to test at least 30.

Protocol
1–2 weeks

B. Protocol: Screening pSingle-tTS-shRNA Clones for Induction

1. Once the picked clones are sufficiently expanded to split into 3 wells of a 6-well plate, seed about 1/3 of the total of a clone into a single well of a "stock plate". These cells will be propagated depending upon the results of the screening assay.
2. Divide the remaining 2/3 of the cells between two wells of a 6-well plate, and to one of the wells, add Dox at the concentration found to give maximal activity in the pilot experiment described in Section VII. For example, suitable concentrations for HeLa and HEK 293 cells are 300 ng/ml and 1,000 ng/ml, respectively. You should also prepare sufficient well(s) of untransfected cells as controls for normal levels of gene expression (+/– Dox).

   NOTE: Be sure to culture the cells using Tet System Approved FBS or serum shown to be free of tetracycline activity.
3. Incubate the cells for 48–72 hr.
4. Harvest the 2 test wells for each clone and controls, and assay for shRNA-mediated gene suppression using the gene-specific method of choice.
5. Clones that exhibit the highest overall shRNA expression (highest level of gene supression) in the presence of Dox and show little or no background supression in the absence of Dox, should be selected for propagation and further testing.
6. Freeze stocks of each relevant clone as soon as possible to provide a renewable source of cells.

IX. Working with Stable Inducible RNAi Cell Lines

Tetracyline-controlled systems have been established successfully in many cell types, as well as transgenic mice, rats, plants, and yeast. The key to generating successful stable cell lines is to pick and carefully screen a reasonable number of clones (we usually pick at least 30 clones at each step). Clonal variation in expression of both the tTS silencer and the shRNA construct is affected by the genomic integration site, which can be readily mitigated simply by screening more clones to find an optimal expressor.

A. Determination of Effective Concentrations of Dox

The concentrations of Dox listed throughout this protocol are approximate. The optimal concentration may vary with different cell lines and with different antibiotic lots. In general, full activation of shRNA expression with stable cell lines can be obtained with 10 ng/ml–1 µg/ml Dox. Perform a dose-response curve similar to the experiment shown in Figure 6 (Section VII).

B. Loss of Regulation

On occasion, well-characterized double-stable cell lines can appear to lose their responsiveness to Dox. This can occur after changing lots of serum and may be due to Tc contamination. You can eliminate Tc contamination problems by using the Clontech's Tet System Approved FBS provided with the Inducible RNAi System. This serum has been functionally tested with the Tet Systems to ensure against possible Tc contamination. Additional FBS can be purchased separately (Cat. Nos. 631105, 631101, 631107 & 631106). If you observe a sudden loss of responsiveness, check your serum by performing a dose-response curve as described in Section VII.A of the Tet-Off® and Tet-On® Gene Expression Systems User Manual (PT3001-1). You can also try replating and washing the cells 3 hr later to remove any residual antibiotic that may be interfering with induction control (Rennel & Gerwins, 2002).

X. Troubleshooting Guide

TABLE I. TROUBLESHOOTING GUIDE FOR THE KNOCKOUT SINGLE VECTOR INDUCIBLE RNAI SYSTEM

Description of Problem	Explanation	Solution
Problems with oligonucleotide cloning	Incompatible ends on the oligos	Confirm that the 5′ ends of the top and bottom annealed shRNA oligos contain Xho I and Hind III overhangs, respectively.
	Ineffective oligo annealing	Verify that the top and bottom strand sequences are correct and complementary. Ensure that equimolar amounts of oligos were in the annealing reaction. It may be necessary to increase the denaturation temperature prior to slow cooling and annealing.
	Oligos are not full-length	Verify oligo size on 12% polyacrylamide gel. Gel purify if necessary, or order gel-purified oligos.
	Suboptimal oligo concentration in ligation reaction	Verify concentration of the annealed oligos used for ligation. Perform ligations containing 5- to 10-fold range in oligo concentration.
	Inactive ligase or buffer	Check ligation reaction with a control vector and fragment. Ligation requires ATP in buffer.
	Suboptimal competent cells	Use Fusion-Blue Competent Cells. Check transformation efficiency using a test plasmid. Competency should be >1 x 10^8 cfu/µg.
Poor transfection efficiency	Poor DNA purity	Ensure DNA purity by preparing all plasmids for transfection using a NuceoBond Plasmid Kit or NucleoBond Xtra Kit. A CsCl gradient can also be used.
	Ineffective transfection method or conditions	The efficiency of transfection depends primarily on the cell line. Optimizing conditions for each cell type is crucial for consistent transfections. Determine the optimal: 1) amount of transfection reagent; 2) amount of pure DNA; 3) ratio of transfection to DNA; 4) cell density; 5) transfection incubation time; 6) medium conditions. Consult your transfection system user manual for more information and strategies.
	No detectable gene silencing	Suboptimal mRNA target sequences or ineffective shRNAs can be interpreted as poor transfection efficiency, and vice versa. See "Poor knockdown efficiency".
Poor knockdown efficiency	Suboptimal cell clone used	Problems with poor tTS or shRNA expression, or weak knockdown are often solved by screening more cell clones to ensure that a clone is selected that harbors a favorable vector integration site and an optimal expression profile.
	Suboptimal mRNA target sequence	Test at least 4 shRNA sequences for optimal gene silencing. Sequences and constructs should possess the qualities described in Appendix A. Large scale functional screening of shRNA sequences is available with Knockout Clone & Confirm PCR Kits. The shRNA sequences so tested are not readily transferrable to pSingle-tTS-shRNA.
	Weak gene suppression and/or leaky background	Serum in medium is contaminated with tetracycline. Use Tet System Approved FBS or check your serum by performing a dose response curve.

X. Troubleshooting Guide continued

TABLE I. TROUBLESHOOTING GUIDE FOR THE KNOCKOUT SINGLE VECTOR INDUCIBLE RNAI SYSTEM

Description of Problem	Explanation	Solution
Loss of inducible regulation	Poor gene suppression/ leaky background	Serum in medium is contaminated with tetracycline. Use Tet System Approved FBS or check your serum by performing a dose response curve.
	Viral promoter inactivation	• Clonal cell line is not pure, and culture is overgrown by non-responding cells. • Viral promoters are subject to switching off or reduced activity due to methylation. • Solution is to subclone the cell line and screen again, or thaw an aliquot of frozen stock of the transfected cells.

XI. References

You can access extensive technical resources and information (including bibliographies) on RNAi and Tet Systems at their respective product pages at www.clontech.com. Clontech offers a collection of online tools to assist you with shRNA oligonucleotide sequence design.

Clontech's Tet Systems were developed in cooperation with Dr. Bujard and his colleagues at the Center for Molecular Biology in Heidelberg (ZMBH). Up-to-date information on the Tet system technology and licensing issues can be found at the site maintained by TET Systems Holding GmbH & Co KG at:

www.tetsystems.com/

Please note that Clontech is not responsible for the information on, or the maintenance of, these sites.

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Appendix A: shRNA Target Sequence Requirements

This section describes the process of identifying likely target sequences and designing the corresponding shRNA oligonucleotides. See Table I for examples of target sequences used to successfully disrupt expression of the cognate genes. Comprehensive online tools to assist you with shRNA oligonucleotide design can be found at http://bioinfo2.clontech.com/rnaidesigner/. Note that the resulting upper and lower strand oligonucleotides should have XhoI and HindIII 5′-overhangs, respectively, for cloning into the pSingle-tTS-shRNA vector.

Select target sequences of 19 nucleotides having the following characteristics:

1. Do not select sequences within the 5' and 3' untranslated regions (UTRs), nor regions within 75 bases of the start codon. These may be richer in regulatory protein binding sites (Elbashir et al., 2001). UTR-binding proteins and/or translation initiation complexes may interfere with binding of the RISC.
2. Do not select sequences that contain a consecutive run of 3 or more thymidine residues; a poly(T) tract within the sequence can potentially cause premature termination the shRNA transcript.
3. The GC content should be between 40% and 60%; a GC content of approximately 45% is ideal.
4. Sequences that have at least 3 A or T residues in positions 15–19 of the sense sequence also appear to have increased knockdown activity.
5. Each oligonucleotide sequence should have minimal secondary structure and be without long base runs, both of which can interfere with proper annealing. Eliminate candidate sequences that display these characteristics.
6. Compare the remaining candidate sequences to an appropriate genome database to identify sequences that are specific for the gene of interest and show no significant homology to other genes. Candidate sequences that meet these criteria are potential shRNA target sites.
7. Test at least 4 shRNAs per gene. It may help to choose shRNA targets that are distributed along the length of the gene sequence to reduce the chance of targeting a region that is either highly structured or bound by regulatory proteins.

TABLE II. EXAMPLES OF PUBLISHED TARGET SEQUENCES[a]

Gene	Target sequence[b]	Sense sequence	Antisense sequence	Reference
β-actin	AATGAAGATCAAGATCATTGC	TGAAGATCAAGATCATTGC	GCAATGATCTTGATCTTCA	Harborth *et al.*, 2001
Bcr-abl	AAGCAGAGTTCAAAGCCCTT	GCAGAGTTCAAAGCCCTT	AAGGGCTTTGAACTCTGC	Scherr *et al.*, 2002
hRad9	AAGTCTTTCCTGTCTGTCTTT	GTCTTTCCTGTCTGTCTTT	AAAGACAGACAGGAAAGAC	Hirai & Wang, 2002

[a] Sequences are shown for top strand oligo design. All sequences shown 5' to 3'. Bottom strand oligo design (not shown) is the complementary sequence to the top strand.
[b] Identified from gene coding sequence.

Appendix B: Vector Information

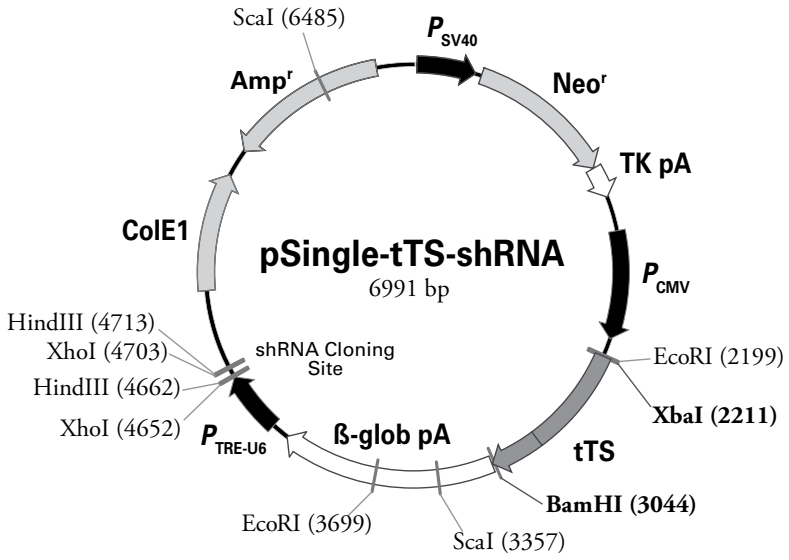

Figure 8. pSingle-tTS-shRNA Vector Map. The pSingle-tTS-shRNA Vector expresses the tetracyline (Tc)-controlled transcriptional suppressor (tTS) which, in turn, controls expression of an shRNA sequence inserted in the shRNA cloning site. The tTS protein is a fusion of the Tet repressor protein (TetR) and the KRAB-AB silencing domain of the Kid-1 protein (SD^{Kid-1}), a powerful transcriptional suppressor (1, 2). In the *absence* of doxycycline (Dox), a Tc derivative, tTS binds to the *tet*O sequences in the modified Tet-responsive Pol III hybrid promoter ($P_{TREmod/U6}$) of the shRNA expression cassette and blocks expression of the shRNA. As Dox is added to the culture medium, tTS dissociates from $P_{TREmod/U6}$ to allow Pol III-mediated transcription of the shRNA, resulting in supression of target gene activity in a highly dose-dependent manner. pSingle-tTS-shRNA also contains a bacterial origin of replication and the Amp^r gene for propagation and selection in *E. coli*; and the neomycin^r gene for selection of stable transformants in mammalian cells.

Appendix B: Vector Information

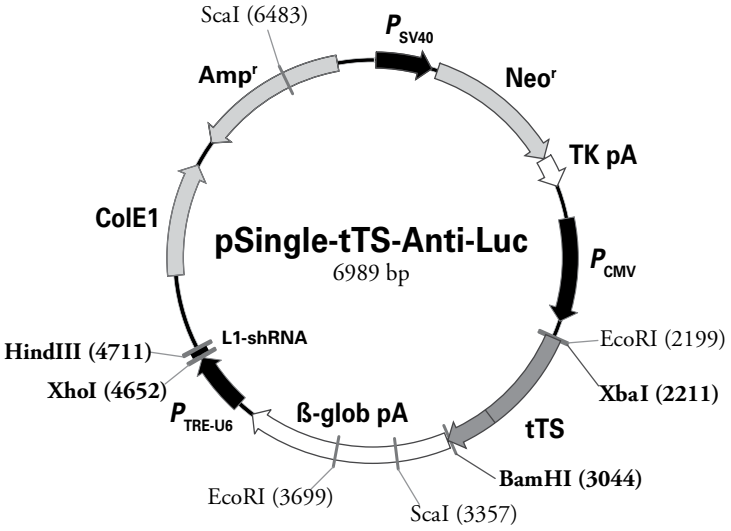

Figure 9. pSingle-tTS-Anti-Luc Vector Map. The pSingle-tTS-Anti-Luc Vector expresses the tetracyline (Tc)-controlled transcriptional suppressor (tTS) which, in turn, controls expression of an anti-luciferase shRNA sequence (L1) inserted in the shRNA cloning site. The tTS protein is a fusion of the Tet repressor protein (TetR) and the KRAB-AB silencing domain of the Kid-1 protein (SD^{Kid-1}), a powerful transcriptional suppressor (1, 2). In the *absence* of doxycycline (Dox), a Tc derivative, tTS binds to the *tet*O sequences in the modified Tet-responsive Pol III hybrid promoter ($P_{TREmod/U6}$) of the shRNA expression cassette and blocks expression of the shRNA. As Dox is added to the culture medium, tTS dissociates from $P_{TREmod/U6}$ to allow Pol III-mediated transcription of the anti-luciferase shRNA, resulting in supression of luciferase gene activity in a highly dose-dependent manner. pSingle-tTS-Anti-Luc also contains a bacterial origin of replication and the *E. coli* Amp^r gene for propagation and selection in bacteria; and the neomycin^r gene for selection of stable transformants in mammalian cells.

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